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PRINCIPAL INVESTIGATOR: Paul D. Walden, Ph.D.

CONTRACTING ORGANIZATION: New York University School of Medicine
New York, NY 10016

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Paul D. Walden, Ph.D.

E-Mail: paul.walden@nyu.edu

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14. ABSTRACT

Based on our preliminary findings, the working hypothesis tested in this study was that the tumor suppressive activity of the BTG2 protein is diminished as an early event in prostate carcinogenesis due to increased proteasomal degradation, leading to compromised cell cycle regulation and increased cell invasion. During this study we showed that BTG2 protein expression is lost as an early event in prostate carcinogenesis and that prostate cancer cells degrade BTG2 at a greater rate than non-cancerous prostate cells. Steady state levels of BTG2 during the cell cycle appear to regulated by changes in ubiquitination (consistent with proteasomal degradation) and not by changes in the level of the deubiquitinating enzyme USP4. BTG2 has a predominantly nuclear localization consistent with its antiproliferative function, but at 4 hours following growth stimulation of quiescent cells, BTG2 is transiently sequestered in the nucleolus coinciding with transiently reduced rates of degradation (BTG2 is synthesized at similar rates during the cell cycle). Forced expression in PC-3 prostate cancer cells (which do not normally express detectable levels of BTG2) results in increased cell attachment to gelatin, fibronectin and type I collagen substrates. Forced expression in PC-3 prostate cancer cells reduces cell invasion through an extracellular matrix.

15. SUBJECT TERMS
Tumor suppressor gene, prostate cancer, tumor biology

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INTRODUCTION: The B-cell translocation gene-2 (BTG2) was originally identified as a primary response gene induced by growth factors and tumor promoters and has been linked to diverse cellular processes such as cell-cycle progression, differentiation or apoptosis.[1] The BTG2 protein has also been shown to interact with the Pop2/Caf1 deadenylase and to be a general activator of mRNA deadenylation.[2] Our interest in BTG2 stemmed from the observation that this protein appears to function in the prevention of malignant transformation of prostate epithelial cells.[3,4] Forced expression of BTG2 in PC-3 prostate cancer cells (which do not normally express detectable levels of BTG2) is associated with significantly reduced rates of both cell proliferation and tumorigenicity.[3,4] $BTG2^{-/-}$ cells continue to proliferate in the presence of DNA damaging agents[5] predisposing cells to the accrual of genetic damage. Steady state levels of BTG2 protein are regulated posttranslationally and increased rates of proteasomal degradation may account for loss of BTG2 protein expression during prostate carcinogenesis.[3] We have shown that ectopic expression of BTG2 in BTG2 null PC-3 prostate cancer cells causes reduced expression of cyclin D1, cyclin B, and thymosin β4 indicative of a role in reducing cell migration as well as blocking cell cycle progression. Consistent with this finding**,** forced expression of BTG2 in PC-3 cells was associated with reduced local tumor growth and absence of metastases following orthotopic injection in the prostates of nude mice. These findings formed the basis for our working hypothesis that the tumor suppressive activity of BTG2 is lost as an early event in prostate carcinogenesis due to increased proteasomal degradation, leading to compromised cell cycle regulation, increased cell invasion and cancer progression. We proposed 3 tasks to test this hypothesis: Task 1: Define BTG2 expression during the morphological transition of normal prostate epithelial cells into prostate cancer precursors (PIA and HGPIN); Task 2: Compare the mechanism of BTG2 protein degradation in non-cancerous and cancerous prostate tissue and epithelial cells and Task 3: Determine the effects of BTG2 on prostate cell attachment and invasion.

BODY: Research accomplishments associated with the approved *Statement of Work* are described below.

Task 1: Define BTG2 expression during the morphological transition of PIA (proliferative inflammatory atrophy) into HGPIN (high-grade prostatic intraepithelial neoplasia) (months 1-5).
All the studies in task 1 were completed.

a. Identify specimens from our archival tissue resource that contain adjacent normal prostate epithelium, PIA and HGPIN (month 1).
This was completed. We identified 50 specimens that were used in subsequent parts of task 1.

b. Cut tissue sections from these specimens for immunohistochemistry (months 1-2).
This was completed. The tissue sections were used in subsequent parts of task 1.

c. Perform immunohistochemistry on adjacent tissue sections to detect (i) the Ki-67 proliferation antigen, (ii) apoptotic nuclei, (iii) BTG2, (iv) high-molecular weight (basal cell) cytokeratin, (v) low-molecular weight (luminal cell) cytokeratin, (v) p27Kip1, (vi) bcl-2 and (vii) COX-2 (months 2-4).
This was completed. The analysis was proposed as Task 1e below.

d. Record changes in the pattern of BTG2 expression, at the cellular (basal and luminal) and sub-cellular (cytoplasmic, nuclear and nucleolar) levels during the morphological transition of normal prostate epithelium to PIA and HGPIN (months 3-5).
BTG2 protein was expressed in the basal (high molecular weight cytokeratin positive) layer of normal prostate epithelium (NPE). Approximately 50% of the basal cells stained positive for BTG2 in normal glands (Figure 1A). Expression was predominantly nuclear. In PIA (which some investigators believe to be a very early prostate cancer lesion that predisposes to HGPIN), BTG2 was expressed in both cell layers (high- and low- molecular weight cytokeratin positive cells)(Figure 1B). In HGPIN (the earliest generally recognized prostate cancer precursor) BTG2 expression was consistently either absent or present at very low levels (Figure 1C). Therefore BTG2 protein expression is activated in PIA but inactivated during the transition to HGPIN. This has important implications in disease progression.

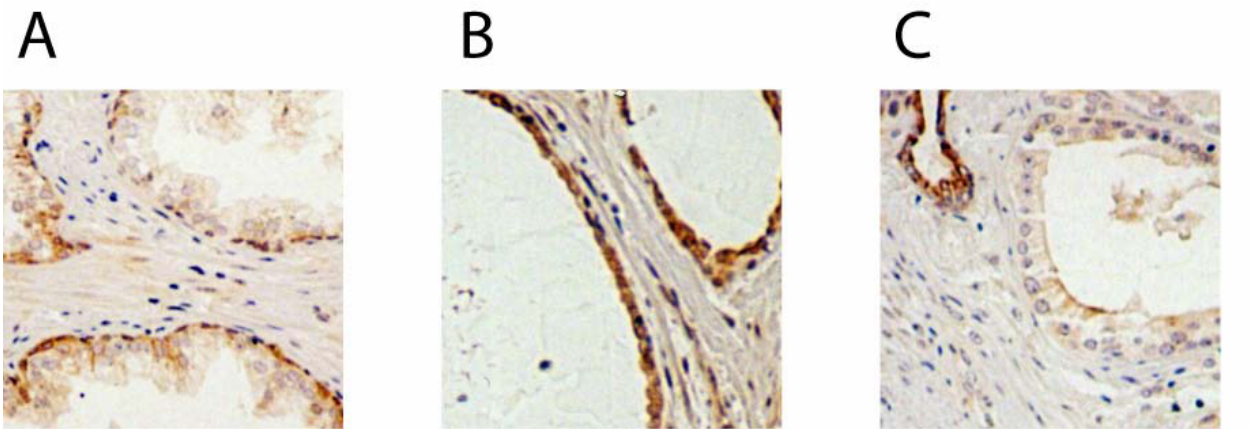

Figure 1. Representative immunohistochemical Images of BTG2 expression in prostate. Tissue was stained under identical conditions as part of a microarray. Normal prostate epithelium (NPE) (A); PIA (B); HGPIN with adjacent PIA (C).

e. Analyze the relationship between changes in expression of BTG2, p27Kip1, bcl-2 and COX-2 (months 3-5).
Ki-67 staining used as a marker of proliferation was low in normal prostate epithelium (NPE) and significantly higher in both PIA and HGPIN (Table 1).

Lesion	Proliferation, % of cells ± SEM	*P* value, vs NPE
NPE	0.29 ±0.08	-
PIA	2.64 ± 0.72	.0045
HGPIN	4.66 ± 1.13	.0042

Table 1. Proliferative indices for NPE, PIA and HGPIN. The percentage of cells ± SEM staining positive for the proliferation associated antigen Ki-67 are shown.

The respective apoptotic indices (determined using the TUNEL assay) are shown in Table 2. The apoptotic rate was not significantly different comparing NPE and PIA, but HGPIN had a significantly higher rate of apoptosis compared to NPE.

Lesion	Apoptosis, % of cells ± SEM	*P* value, vs NPE
NPE	0.022 ± 0.006	-
PIA	0.024 ± 0.008	NS
HGPIN	0.19 ± 0.06	.0122

Table 2. Apoptotic indices for NPE, PIA and HGPIN. The percentage of cells ± SEM staining positive by the TUNEL assay are shown.

These results are consistent with the notion that PIA is a regenerative lesion (high proliferation, low apoptosis) and that HGPIN is a rapidly turning over lesion (high proliferation and high apoptosis). Consistent with earlier reports, we found p27 expression to be significantly decreased, and bcl-2 expression to be significantly increased in PIA and HGPIN (Figure 2). Similar to the situation with BTG2, COX-2 expression was upregulated in PIA, but not in HGPIN.

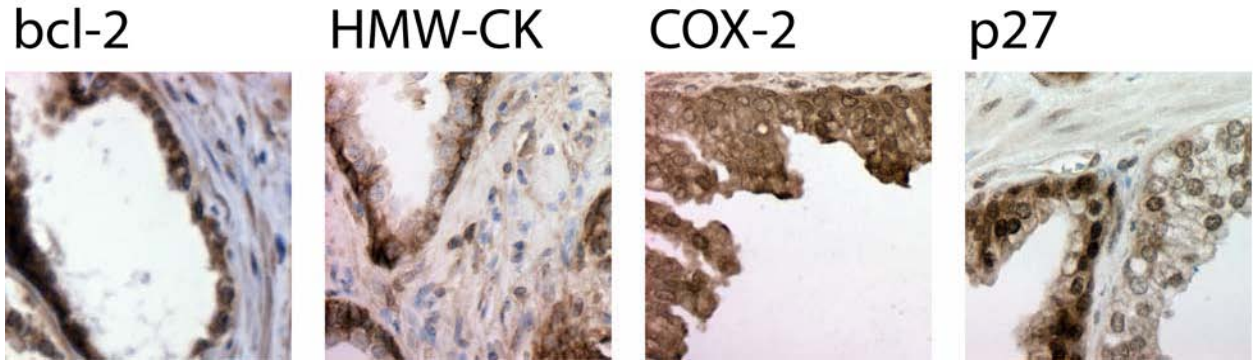

Figure 2. Representative images showing immunohistochemical staining for bcl-2, basal cell cytokeratin, COX-2 and p27 in prostate tissue. Images show: Bcl-2 expression in PIA; high-molecular weight cytokeratin (HMW-CK) staining of basal cell layer in NPE. COX-2 expression in glands adjacent to PIA; p27 expression in benign glands adjacent to HGPIN where staining is absent/low.

Task 2: Compare the mechanism of BTG2 protein degradation in non-cancerous and cancerous prostate tissue and epithelial cells (months 5-26).
This was completed.

a. Histologically characterize and isolate adjacent regions of non-cancerous and cancerous prostate tissue (months 5-9).
This was completed.

b. Determine the rates of BTG2 protein degradation in extracts of non-cancerous prostate tissue specimens and prostate cancer and determine the contribution of the ubiquitin-proteasome pathway to the degradation process (months 5-9).
This was completed. We have shown that extracts of cancer tissue degrade exogenously added BTG2 as a greater rate than extracts of adjacent non-cancer tissue and that this process could be inhibited by the specific proteasomal inhibitor lactacystin (Figure 3).

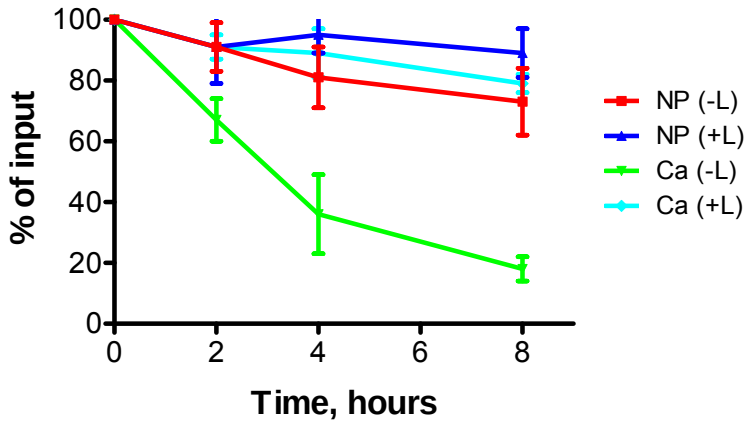

Figure 3. Rates BTG2 degradation in extracts of cancer tissue and adjacent non-cancer tissue. The percentage of input BTG2 remaining after 0, 2, 4 and 8 hours of incubation with cell-free extracts prepared from noncancerous (NP) or cancerous (Ca) prostate tissues was determined by SDS-PAGE and densitometric scanning of gels. Experiments were conducted in the presence (+) or absence (-) of lactacystin (L).

c. Establish and maintain primary cultures of prostate epithelial cells and the LNCaP and PC-3 cell lines for study (months 10-26).
This was completed.

d. Determine the rates of BTG2 protein degradation in extracts of non-cancerous and cancerous prostate epithelial cells and prostate cancer cell lines and determine the contribution of the ubiquitin-proteasome pathway to the degradation process (months 10-14).
This was completed. Extracts prepared from primary cultures of cancerous human prostate epithelial cells degrade BTG2 at a significantly greater rate than extracts from primary cultures of non-cancerous human prostate epithelial cells (Table 3).

Half-life, minutes		*P* value
Non-Cancerous	Cancerous	
27.3 ± 2.1	16.5 ± 1.2	.0017

Table 3. Half–life of BTG2 protein in extracts of primary cultures of cancerous and non-cancerous human prostate epithelial cells

Rates of BTG2 protein degradation in extracts of LNCaP and PC-3 prostate cancer cells were similar to those in cancerous prostate epithelial cells. In all cell lines we were able to inhibit BTG2 degradation by addition of the proteasome inhibitor lactacystin. These findings suggest that the discrepancy between steady sate levels of BTG2 protein comparing cancerous and non-cancerous prostate epithelial cells is due at least in part to increased proteasomal degradation in cancer.

e. Determine the rates of BTG2 protein degradation during the cell-cycle in extracts of non-cancerous and cancerous prostate epithelial cells and prostate cancer cell lines (months 14-18).
This was completed, however the studies took longer than originally planned for several reasons. The major reason was due to technical difficulties with cell synchronization. Initially, the cells

could not be reliably synchronized in the commercially available growth medium we typically use for epithelial cells (Keratinocyte SFM). They would not grow or synchronize at low densities necessary to conduct these studies. To resolve this issue we switched to the original "home-made" complex medium we had previously used to grow these cells.[6] After resolving these issues, we were able to get reproducibly synchronized cells by making them quiescent in the absence of EGF and allowing the cells to enter the growth cycle by addition of EGF. We were then able to get very reproducible BTG2 staining during the cell cycle using these conditions (Figure 4).

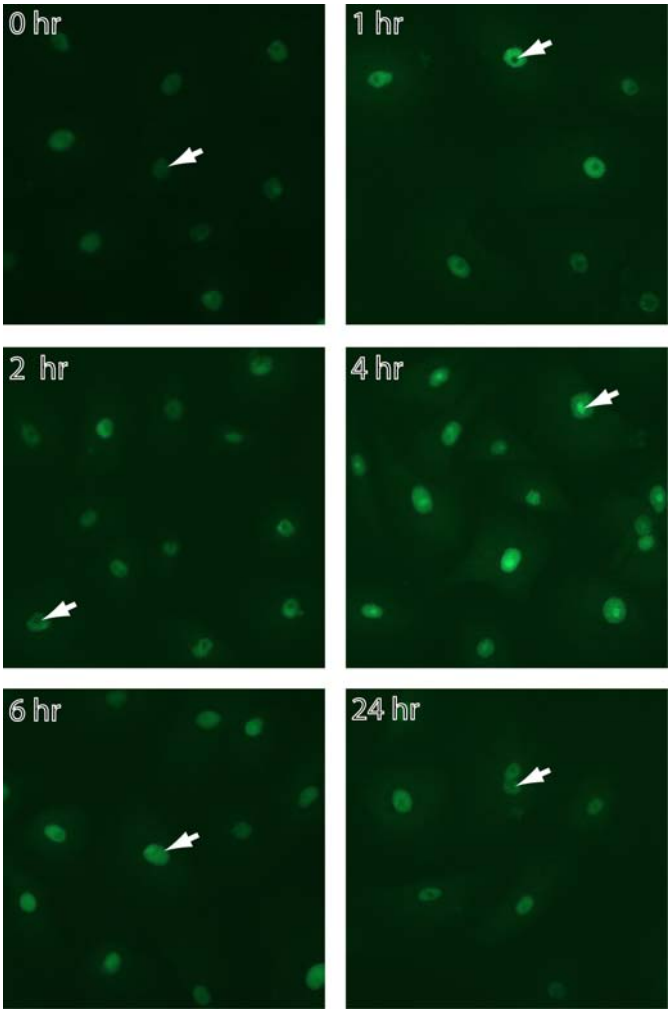

Figure 4. **Subcellular localization of BTG2 protein during the cell cycle of primary cultures of non-cancerous human prostate epithelial cells**. BTG2 protein was localized in by immunofluorescence microscopy in primary cultures of non-cancerous human prostate epithelial cells that were quiescent (0 hr) or had been stimulated to enter the growth cycle by addition of EGF (1 hr to 24 hr). Arrows show nucleolus.

BTG2 shows predominantly nuclear localization throughout the cell cycle, entirely consistent with its localization in human prostate and breast tissues. Interestingly and unexpectedly, prior to 4 hr BTG2 does not accumulate in the nucleolus, but at 4hr (corresponding with the S-phase of the cell cycle) there is intense nucleolar staining for BTG2. After 4 hours, until 12 hours BTG2 is localized throughout the nucleus and nucleolus in the majority of cells. After 12 hours, nucleolar

staining decreases and by 24 hours nucleolar staining has diminished. The BTG2 localization pattern was very similar in cancer cells, except the signal was much weaker (data not shown). Thus differences in localization cannot account for differences in BTG2 function in cancer. In HGPIN, when BTG2 protein levels decline weak nucleolar staining for BTG2 persisted. Nuclear localization of BTG2 is consistent with its antiproliferative function, but given its nucleolar localization, BTG2 might influence some aspect of ribosome biosynthesis.

The mechanism underlying the original observation that BTG2 was an antiproliferative gene induced in response to growth promoters remained elusive for several years until it was demonstrated that BTG2 can induce apoptosis in response to EGF in U937 tumor cells via a p53 independent mechanism that involves phosphorylation of BTG2 by Erk1/2 which in turn induces BTG2 to bind to Pin-1.[7] BTG2 is therefore able to inhibit cell cycle progression and malignant transformation by multiple mechanisms, which is entirely consistent with our working hypothesis.

f. Determine if there are differential rates of BTG2 protein synthesis in addition to differential rates of protein degradation (months 18-23).
This was completed.

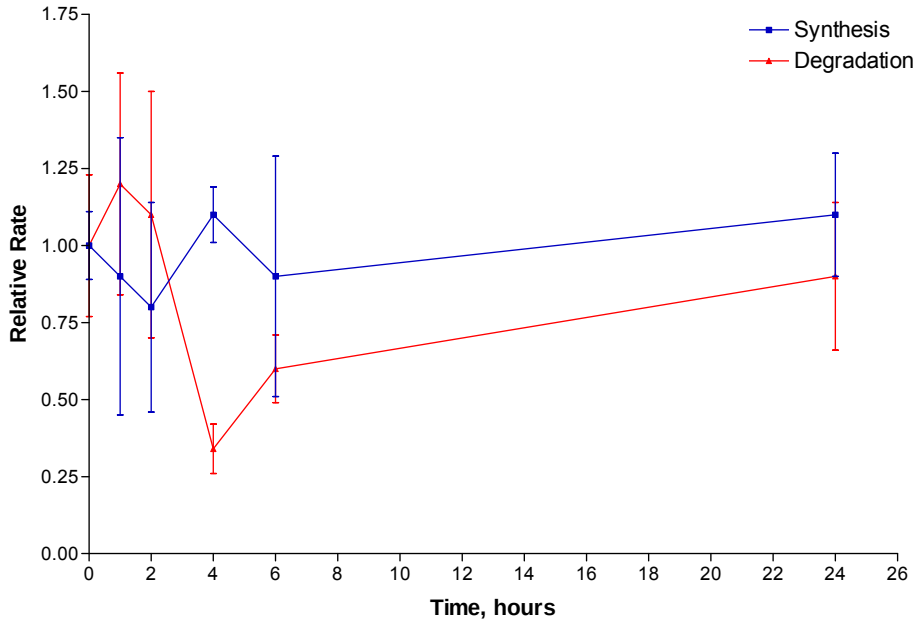

Figure 5. Rates of BTG2 protein synthesis and degradation in primary cultures of prostate epithelial cells. Rates of synthesis and degradation were determined by pulse-chase labeling with ^{35}S-methionine followed by immunoprecipitation with anti-BTG2 antibodies and SDS-PAGE. Quantification was determined by phosphorimager analysis of the dried gel. Rates were determined relative to quiescent cells which were assigned the arbitrary value of 1.

Rates of BTG2 protein synthesis were not markedly different throughout the cell cycle, however rates of degradation were significantly reduced at 4 hr coincident with nucleolar localization of BTG2 seen in Figure 5. Similar results were seen in cancer cells although the reduced degradation at 4 hr was not as pronounced (data not shown). Thus are marked differences in the cell cycle dependent degradation and subcellular accumulation of BTG2.

g. Determine the extent of ubiquitination of BTG2 in extracts of non-cancerous and cancerous prostate epithelial cells and prostate cancer cell lines (months 23-25).
This was completed. We modified the originally proposed in vitro ubiquitination assay due to technical difficulties we experienced. Recombinant [^{35}S]-labeled BTG2 protein was synthesized in *E. coli* by incubating the bacteria in sulfate-free medium containing [^{35}S]-sulfate. The labeling phase was chased with cold sulfate to ensure production of full-length BTG2 protein products. The [^{35}S]-labeled BTG2 product was purified from bacterial lysates by ion-exchange and size exclusion chromatography. Aliquots of [^{35}S]-labeled BTG2 were incubated with proteasome inhibitors and extracts of prostate epithelial cells prepared at quiescence and at various time periods into the growth cycle. Reactions were quenched by boiling in SDS-PAGE sample buffer and the products were examined by SDS-PAGE and fluorography. We determined that the extent of ubiquitination of BTG2 was cell-cycle dependent in all cell-lines examined. The results for primary cultures of non-cancerous human prostate epithelial cells are shown in Figure 6. BTG2 ubiquitination was undetectable by this assay in quiescent cells and in cells that had been stimulated to enter the growth cycle for 1 hour.

Ubiquitination of BTG2 was seen to gradually increase in cells at 2 hours through 24 hours into the growth cycle and is generally consistent in explaining the levels of BTG2 protein seen during the cell cycle. However, our findings from the previous reporting period also indicate that BTG2 is sequestered into the nucleolus at 4 hr following entry into the cell cycle and that the protein accumulates transiently in this organelle (possibly due to reduced degradation in the nucleolus). These findings indicate that the regulation of steady state levels of BTG2 protein during the cell cycle is more complex that we originally anticipated and suggests an additional function for BTG2 in the nucleolus.

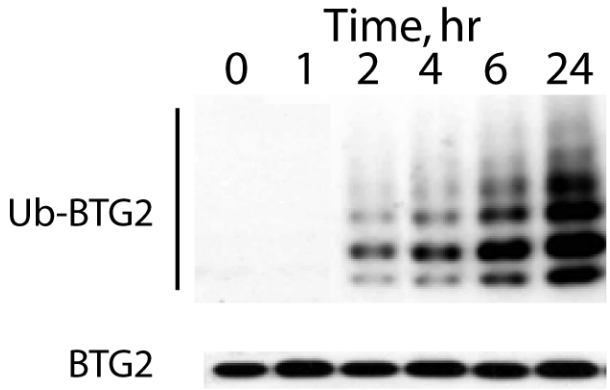

Figure 6. Extent of BTG2 protein ubiquitination during the cell cycle of primary cultures of non-cancerous human prostate epithelial cells. [^{35}S]-labeled BTG2 protein was incubated with extracts of primary cultures of non-cancerous human prostate epithelial cells that were quiescent (0 hr) or had been stimulated to enter the growth cycle by addition of EGF (1 hr to 24 hr). The products were then analyzed by SDS-PAGE and fluorography. The BTG2 and ubiquitinated BTG2 (Ub-BTG2) regions of the same gel are shown (exposure times for the Ub-BTG2 region were longer).

h. Determine the levels of de-ubiquitinating enzyme Unp-1 in extracts of non-cancerous and cancerous prostate epithelial cells and prostate cancer cell lines (months 24-26).
This has been completed. During the tenure of this award, antibodies to the human ubiquitin specific protease USP4 became commercially available from Bethyl Laboratories (Montgomery, TX). USP4 is the human analog of murine Unp-1. Levels of USP4/Unp-1 did not change

significantly during the cell cycle (Figure 7). Taken together these findings suggest that BTG2 protein levels during the cell cycle are regulated by ubiquitination and not by alterations in the level of the ubiquitin specific protease USP4.

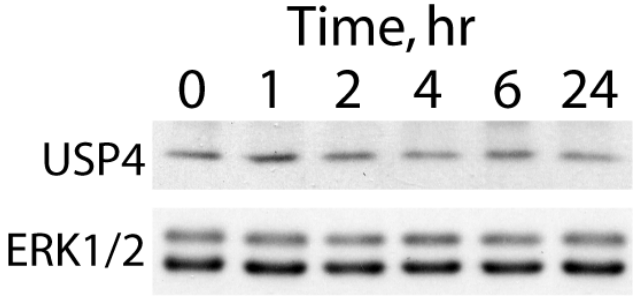

Figure 7. Levels of the ubiquitin specific protease USP4 during the cell cycle of primary cultures of non-cancerous human prostate epithelial cells. Extracts of primary cultures of non-cancerous human prostate epithelial cells that were quiescent (0 hr) or had been stimulated to enter the growth cycle by addition of EGF (1 hr to 24 hr) were analyzed by SDS-PAGE and immunoblotting using antibodies to USP4 and p42/p44 MAPK (as loading control)

Task 3: Determine the effects of BTG2 on prostate cell attachment and invasion (months 26-36).
Due to technical difficulties these studies took much longer than planned. The PC-3/Retro-Off BTG2 cells that express BTG2 under the control of an inducible tetracycline promoter were prepared before the original proposal was submitted. These cells were frozen down for use in task 3 of the proposal, however the cells did not maintain adequate levels of inducible expression upon thawing. These cells therefore had to be rederived in order to initiate Task 3. These studies were completed during a no-cost extension of this award.

a. Maintain cultures of PC-3/Retro-Off BTG2 cells that express BTG2 under the control of an inducible tetracycline promoter (months 26-36).
This was completed. We rederived PC-3/Retro-Off BTG2 cell lines that expressed undetectable levels of BTG2 in the presence of the transcriptional repressor doxycyline, and that expressed detectable levels of BTG2 when doxycycline was removed from the medium. BTG2 expression was determined by both immunofluorescence microscopy and immunoblotting. Cells were routinely maintained in regular cell culture medium for PC-3 cells and 0.5 μg/ml of the transcriptional repressor doxycyline. Prior to seeding for the experiments below, cells were trypsinized and washed in medium alone (+BTG2) or in medium containing 0.5 μg/ml doxycyline (+BTG2).

b. Determine whether BTG2 expression in PC-3/Retro-Off BTG2 cells increases cell attachment to different extracellular matrices (months 26-31).
This was completed. Forced expression of BTG2 in PC-3 cells was associated with significantly higher cell attachment to all 3 substrates tested (gelatin, fibronectin and type I collagen) (Table 4).

Substrate	BTG2 Expression*	Cell count (mean ± SEM)†	*P* value
Gelatin	–	19 ± 2.2	.029
	+	32 ± 4.6	
Fibronectin	–	18 ± 1.4	.033
	+	28 ± 3.8	
Type I Collagen	–	16 ± 1.8	.038
	+	25 ± 3.3	

Table 4. Effects of BTG expression on PC-3 cell adhesion to gelatin, fibronectin and type I collagen. Subconfluent cultures of PC-3/Retro-Off BTG2 cells were treated with either vehicle (to induce expression of BTG2) or the transcriptional repressor doxycycline (0.5 µg/ml) and then seeded in 6-well plates that had been coated with either gelatin, fibronectin or type 1 collagen. Cells were allowed to attach for 10 minutes at 37°C, rinsed with Hanks buffered saline, fixed with methanol at -20°C, and counted using computer assisted image analysis.
*Expression of BTG2 was confirmed by fluorescence microscopy in parallel experiments performed on gelatin, fibronectin or type 1 collagen coverslips. †Average cell counts from 10 different fields for six individual assays were scored.

c. Determine whether BTG2 expression in PC-3/Retro-Off BTG2 cells decreases tumor cell invasion through the extracellular matrix (months 31-36).
This was completed. For the tumor cell invasion assays, we used the BioCoat™ system (BD Biosciences) comprising a 24-well plate with inserts containing a Fluoroblok™ light-tight fluorescent blocking polyethylene terephthalate membrane (8.0 µm pore size) coated with Matrigel™ matrix. The principle of this tumor invasion system is to provide an barrier to non-invasive cells, while at the same time providing an appropriate extracellular matrix for invasive cells allowing these cells pass through the Fluoroblok membrane. Fluorescently labeled cells appearing on the underside of the Fluroblok membrane are quantitated by real-time fluorescence that is directly correlated to cell number (Figure 8).

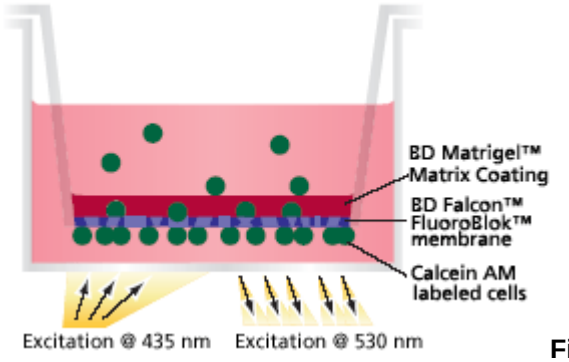

Figure 8. Schematic of the BioCoat system

The protocol originally proposed post-labeling of the cells in the assay. This was subsequently changed to a pre-labeling protocol to allow real-time analysis of the effects of BTG2 expression on PC-3 cell invasion. BTG2 expression reduced cell invasion in this tumor cell invasion system (Figure 9).

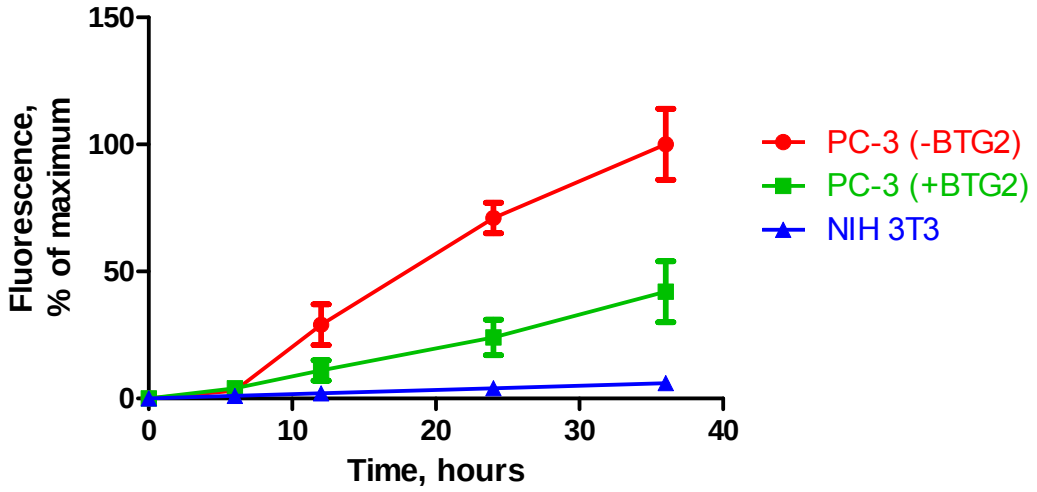

Figure 9. Effects of BTG2 expression on PC-3 cell invasion in real time. BioCoat™ insert plates were prepared by rehydrating the Matrigel coating in PBS for 2 hrs at 37°C. PC-3/Retro-Off BTG2 cells were prelabeled in situ with 1 µM Calcein AM in HBSS/0.1% BSA (±0.5 µg/ml doxycyline) for 30 minutes at 37ºC. Cells were washed with PBS and were plated at a density of 3.75 X 10^4 cells/well in the upper chamber in media alone +1% BSA (no FBS). The bottom chamber of the wells contained media + 10% FBS as chemoattractant. Cells were maintained in either the absence (+BTG2), or presence (-BTG2) of 0.5 µg/ml doxycyline. Plates were incubated at 37°C and analysed at various time points in a fluorescence plate reader using excitation/emission wavelengths of 485/535mn. Non-invasive NIH 3T3 cells were used as control

KEY RESEARCH ACCOMPLISHMENTS

During the entire period of funding we have:

- Shown that BTG2 protein expression is lost as an early event in prostate carcinogenesis consistent with a role as a tumor suppressor
- In work outside the scope of the present study (but using reagents and ideas generated in the present study), shown that BTG2 protein is also lost as an early event in breast cancer[8,]
- Defined the expression pattern of the BTG2 during early prostate tumorigenesis in relation to markers of cell proliferation, apoptosis, and inflammation
- Demonstrated a relationship between loss of expression of p27 and BTG2 in HGPIN and a relationship between elevated expression of COX-2 and BTG2 in PIA
- Shown the half-life of BTG2 to be significantly reduced in cancerous vs non cancerous prostate epithelial cells
- Shown that BTG2 is a nuclear protein throughout the cell cycle and that the protein accumulates in the nucleolus at 4 hr following entry into the cell cycle stimulated with EGF.
- Shown that BTG2 is synthesized at similar rates during the cell cycle, but that degradation is reduced at 4 hr following entry into the cell cycle stimulated with EGF
- Shown that BTG2 protein is increasingly polyubiquitinated after prostate epithelial cells enter the cell cycle
- Shown that BTG2 protein levels are likely regulated during the cell cycle by ubiquitination and not by alterations in the level of the ubiquitin specific protease USP4
- Shown that forced expression in PC-3 prostate cancer cells (which do not normally express detectable levels of BTG2) results in increased cell attachment to gelatin, fibronectin and type I collagen substrates

- Shown that forced expression in PC-3 prostate cancer cells reduces cell invasion through an extracellular matrix

REPORTABLE OUTCOMES

Publications:

- Two manuscripts have been published that indirectly relates to the research in this proposal
    - Kawakubo, H., Carey, J. L., Brachtel, E., Gupta, V., Green, J. E., Walden, P. D., and Maheswaran, S. Expression of the NF-κB-responsive gene BTG2 is aberrantly regulated in breast cancer. *Oncogene*. 2004;23:8310-8319
    - Kawakubo H, Brachtel E, Hayashida T, Yeo G, Kish J, Muzikansky A, Walden PD, Maheswaran S. Loss of B-cell translocation gene-2 in estrogen receptor-positive breast carcinoma is associated with tumor grade and overexpression of cyclin d1 protein. *Cancer Res*. 2006;66:7075-7082
- Much of the work conducted under this award has been written up with the intent of submitting for publication; however I am no longer employed at NYU, or in academia. I became another victim of the funding cuts affecting this profession and I left my position in January 2008. Consequently finding the time to finish these manuscripts is proving very difficult

Abstracts:

- Kim, J. & Walden, P.D. Antiproliferative BTG2 protein down regulates cyclins D1 & B and is degraded at a greater rate in prostate cancer cells." Presented at the 27th Congress of the Société Internationale d'Urologie (SIU). October 3-7, 2004, Honolulu, HI

Other:

- Developed PC-3/Retro-Off BTG2 cell lines that express BTG2 under the control of an inducible promoter
- Developed affinity purified antibodies to BTG2 that have been used by several investigators throughout the world

CONCLUSIONS: Research completed under this award has confirmed our working hypothesis expression of the BTG2 tumor suppressor is lost as an early event in prostate carcinogenesis. Our studies have shown that loss of expression in prostate cancer is at least partly due to increased proteasomal degradation of the protein in these lesions. Our prior studies have shown that BTG2 mRNA levels and translation are not affected during early prostate carcinogenesis, and our present studies indicate that early in prostate carcinogenesis (during the NPE/PIA to HGPIN transition) BTG2 protein is targeted for degradation. Given the similarities between p27 and BTG2 loss during prostate carcinogenesis, it is possible that both proteins are affected by similar degradation mechanisms. While some cancers result from the inheritance of genetic mutations in key genes, it is becoming increasingly apparent that the regulation of key protein levels is significant in tumor initiation and progression. Thus, therapeutic modalities that prevent BTG2 protein degradation might be effective in prostate cancer prevention.

Research completed under this award has shown that there is a general increase in ubiquitination of BTG2 during the cell cycle, but there are no corresponding changes in the expression of the ubiquitin specific protease USP4. Thus increased targeting for degradation appears to be a major method of regulation. The transient sequestration of BTG2 in the nucleolus at 4 hours into the

cell cycle, appears to be an additional regulatory mechanism uncovered during this award. While the general mechanisms for BTG2 degradation are similar in non-cancerous and cancerous prostate cells, they result in lower levels of BTG2 protein in cancer cells.

During this award we have shown that forced expression in PC-3 prostate cancer cells (which do not normally express detectable levels of BTG2) results in increased cell attachment to gelatin, fibronectin and type I collagen substrates. Finally forced expression in PC-3 prostate cancer cells reduces cell invasion through an extracellular matrix. Thus during this award we have proven our working hypothesis as well as uncovering some additional information regarding the mechanism of action of BTG2 during prostate carcinogenesis. Several investigators throughout the world are evaluating the mechanistic role of BTG2 as a tumor suppressor. BTG2 expression has been shown to have a negative impact on carcinogenesis in several solid tumors including prostate,[4] breast,[8,9], kidney,[10] medulloblastoma[11], and liver.[12] Research conducted under this award and by other investigators is therefore revealing that BTG2 has an important role in prevention of carcinogenesis. Ongoing research will further uncover the mechanism of action of this important tumor suppressor.

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PERSONNEL RECEIVING PAY FROM RESEARCH EFFORT

Name	Role on Project
Paul Walden, PhD	PI
Jonathan Melamed	Coinvestigator
James Kim	Technician